

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER

56778 (70820)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

Filed Herewith **10/019743**

INTERNATIONAL APPLICATION NO.

PCT/JP00/04277 ✓

INTERNATIONAL FILING DATE

29/06/2000 ✓

PRIORITY DATE CLAIMED

30/06/1999 ✓

TITLE OF INVENTION

PROCESS FOR PREPARING LH-RH DERIVATIVES

APPLICANT(S) FOR DO/EO/US

Yasuhiro SASAKI

Katsuji SHIMIZU

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☐ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
- ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
- ☒ A copy of the International Search Report (PCT/ISA/210).
- ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
- ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
- ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
- ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
- ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

- Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ Certificate of Mailing by Express Mail
20. ☒ Other items or information:

PCT/RO/101; First page of WO 01/02428; PCT/ISA/210; PCT/IPEA/409; PCT/IB/301; PCT/IB/304; PCT/IB/308;
PCT/IB/332

Docket No. 56778 (70820)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: Y. Sasaki et al.

U.S.S.N.: Not yet assigned

GROUP: Unknown

FILED: Filed Herewith
(Express Mail Label No. EL933049726US)

EXAMINER: Unknown

FOR: PROCESS FOR PREPARING LH-RH DERIVATIVES

.....
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

PRELIMINARY AMENDMENT

Applicants kindly request that the above-identified application be amended as follows:

IN THE CLAIMS

Please amend claim 15, and add new claim 18:

15. (Amended) The process according to claim 13, wherein the LH-RH derivative protected with protective group(s) is represented by the formula

5-oxo-Pro-His-Trp-Ser-Tyr-Y-Leu-Arg(X)-Pro-Z

wherein X indicates a protective group, Y indicates a residue selected from DLeu, DAla, DTrp, DSer(tBu), D2Nal and DHis (ImBzl) and Z indicates NH-C₂H₅ or Gly-NH₂, respectively.

18. (New) The process according to claim 14, wherein the LH-RH derivative protected with protective group(s) is represented by the formula

5-oxo-Pro-His-Trp-Ser-Tyr-Y-Leu-Arg(X)-Pro-Z

wherein X indicates a protective group, Y indicates a residue selected from DLeu, DAla, DTrp, DSer(tBu), D2Nal and DHis (ImBzl) and Z indicates NH-C₂H₅ or Gly-NH₂, respectively.

REMARKS

Claim 15 has been amended and new claim 18 has been added solely to remove multiple claim dependencies. No new matter is presented by virtue of this Amendment.

Applicants respectfully request entry of this Amendment prior to examination.

Early consideration and allowance of the application are earnestly solicited.

Respectfully submitted,



Date: December 28, 2001

John B. Alexander, Ph.D. (Reg. No. 48,399)
Dike Bronstein, Roberts & Cushman
Intellectual Property Practice Group
EDWARDS & ANGELL, LLP
P.O. Box 9169
Boston, MA 02209
(617) 439-4444

BOS2_184331.1

APPENDIX A:
VERSION WITH MARKINGS TO SHOW CHANGES MADE

15. (Amended) The process according to claim 13 [or 14], wherein the LH-RH derivative protected with protective group(s) is represented by the formula
5-oxo-Pro-His-Trp-Ser-Tyr-Y-Leu-Arg(X)-Pro-Z
wherein X indicates a protective group, Y indicates a residue selected from DLeu, DAla, DTrp, DSer(tBu), D2Nal and DHis (ImBzl) and Z indicates NH-C₂H₅ or Gly-NH₂, respectively.

18. (New) The process according to claim 14, wherein the LH-RH derivative protected with protective group(s) is represented by the formula
5-oxo-Pro-His-Trp-Ser-Tyr-Y-Leu-Arg(X)-Pro-Z
wherein X indicates a protective group, Y indicates a residue selected from DLeu, DAla, DTrp, DSer(tBu), D2Nal and DHis (ImBzl) and Z indicates NH-C₂H₅ or Gly-NH₂, respectively.

PROCESS FOR PREPARING LH-RH DERIVATIVES

Technical Field

5 The present invention provides a simple and efficient industrial process for preparing LH-RH derivatives and also provides a process for preparing LH-RH derivatives having a high quality.

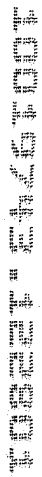
Background Art

10 As for a process for preparing peptides, which are LH-RH derivatives, or salts thereof, JP 50-59370 A (corresponds to U. S. Patent No. 4,008,209) describes the following process as that for preparing a peptide represented by the general formula

15 (Pyr)Glu-His-Trp-Ser-Tyr(or Phe)-X-Leu(or Ile or Nle)-Arg-Pro-NH-R

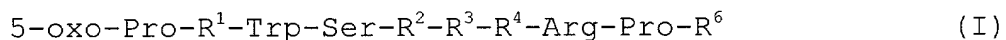
wherein the amino acids indicate L form, unless otherwise specified, X indicates D-Leu, D-Nle, D-Nval, D-Ser, D-Abu, D-Phg, D-Phe or α -Aibu, and R indicates an alkyl group that
20 may have hydroxyl group.

Scheme

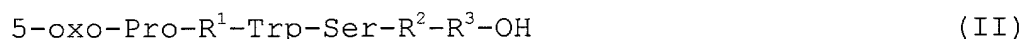


alkylsulfonic acid or a Lewis acid.

Also, WO 97/48726 describes a process for preparing a peptide represented by the general formula



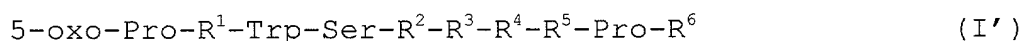
5 wherein the symbols are as defined below, or a salt thereof which is characterized by reacting a peptide represented by the general formula



10 wherein R^1 indicates His, Tyr, Trp or $p\text{-NH}_2\text{-Phe}$, R^2 indicates Tyr or Phe and R^3 indicates Gly or an $\alpha\text{-D-amino}$ acid residue, each of which may have a substituent, respectively, or a salt thereof with a peptide represented by the general formula



15 wherein R^4 indicates Leu, Ile or Nle, R^5 indicates Arg that is protected and R^6 indicates a group represented by the formula Gly-NH-R^7 (wherein R^7 indicates hydrogen atom or an alkyl group that may have hydroxyl group) or by the formula NH-R^8 (wherein R^8 indicates hydrogen atom, an alkyl group
20 that may have hydroxyl group or ureido group ($-\text{NH-CO-NH}_2$), respectively), or a salt thereof to obtain a peptide represented by the general formula



25 wherein the symbols are as defined above, or a salt thereof and then subjecting the thus-obtained peptide (I') to a

deprotection reaction.

In the prior art, many racemic isomers of constituent amino acids are by-produced during preparation steps of LH-RH derivatives from protected peptides thereof.

5 In the prior art technique, the purification of LH-RH derivatives is carried out by column chromatography using a weakly acidic cation-exchange resin or the like, where multi-step chromatography operations are necessary owing to a poor efficacy of removing racemic isomers and the like, so that it is difficult to produce LH-RH derivatives having a higher quality with a good industrial efficiency. The problems to be solved by the present invention is to provide a process for preparing LH-RH derivatives of a high quality in high yields by an industrially very advantageous method, which utilizes a purification procedure that can suppress racemization of constituent amino acids during the preparation of LH-RH derivatives from protected peptides thereof, can effectively remove racemic isomers and other impurities, and can also carry out the purification step simply and effectively.

Disclosure of the Invention

As a result of intensive investigations to solve the above-mentioned problems, the present inventors have achieved improvements in the purification step of LH-RH

derivatives and improvements in the work-up after the deprotection reaction, and have established a method for suppressing racemization of constitutive amino acids during the preparation and purification steps of LH-RH derivatives as well as a purification method that can effectively remove racemic isomers and other impurities and also can carry out the column treatment step simply and effectively to a great extent without using a step for treatment with an ion-exchange resin, thereby having found a process for preparing LH-RH derivatives of a high quality in high yields. As a result of further intensive investigations on the basis of this finding, the present invention has been completed.

That is, the present invention relates to

(1) a process for preparing a LH-RH derivative which comprises subjecting a solution containing the LH-RH derivative to a step for treatment with a methacrylic synthetic adsorption resin and a step for treatment with an aromatic synthetic adsorption resin;

(2) the process as described in the above (1), wherein the LH-RH derivative is a peptide represented by the formula

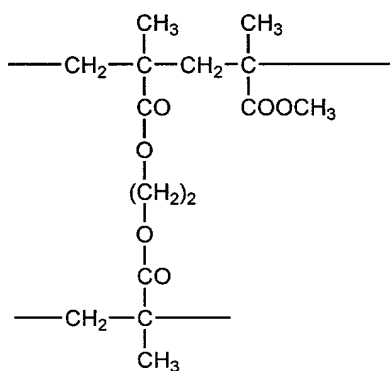
5-oxo-Pro-His-Trp-Ser-Tyr-Y-Leu-Arg-Pro-Z

wherein Y indicates a residue selected from DLeu, DAla, DTrp, DSer(tBu), D2Nal and DHis(ImBzl), and Z indicates NH-

C₂H₅ or Gly-NH₂, respectively, or a salt thereof;

(3) the process as described in the above (1), wherein the LH-RH derivative is a peptide represented by the formula
 5-oxo-Pro-His-Trp-Ser-Tyr-DLeu-Leu-Arg-Pro-NH-C₂H₅
 or its acetate;

5 (4) the process as described in the above (1), wherein said process comprises using a methacrylic synthetic adsorption resin having a repeating unit represented by the formula



;

10 (5) the process as described in the above (1), wherein the aromatic synthetic adsorption resin is a styrene-divinylbenzene synthetic adsorption resin;

(6) the process as described in the above (5), wherein an average particle size of the styrene-divinylbenzene, synthetic adsorption resin is about 60 μm to about 150 μm ;

(7) the process as described in the above (1), wherein said process comprises subjecting a solution containing the LH-RH derivative to the step for treatment with a methacrylic synthetic adsorption resin below about 10°C;

20 (8) the process as described in the above (1), wherein said

process comprises subjecting a solution containing the LH-RH derivative to the step for treatment with an aromatic synthetic adsorption resin at about 10°C to about 20°C;

(9) the process as described in the above (1), wherein said

5 process comprises subjecting a solution containing the LH-RH derivative to the step for treatment with a methacrylic, synthetic adsorption resin, followed by subjecting to the step for treatment with an aromatic, synthetic adsorption resin;

10 (10) the process as described in the above (1), said process comprises passing a solution containing the LH-RH derivative through a resin in the step for treatment with a methacrylic synthetic adsorption resin and then eluting the LH-RH derivative, which is adsorbed on the resin, with an
15 aqueous solution of acetic acid;

(11) the process as described in the above (10), wherein the concentration of an aqueous solution of acetic acid is about 0.01 M to about 0.50 M;

(12) the process as described in the above (1), wherein
20 said process comprises passing a solution containing the LH-RH derivative through a resin in the step for treatment with a methacrylic, synthetic adsorption resin, followed by washing with an aqueous solution of ethanol, and then by eluting the LH-RH derivative that is adsorbed on the resin;

25 (13) the process as described in the above (1), wherein a

solution containing the LH-RH derivative is that obtained by subjecting the LH-RH derivative protected with protective group(s) to a deprotection reaction followed by a neutralization reaction below about 10°C;

(14) the process as described in the above (1), wherein a solution containing the LH-RH derivative is that obtained by subjecting the LH-RH derivative protected with protective group(s) to a deprotection reaction and then a neutralization reaction below about 10°C, followed by subjecting the resulting mixture to extraction of the LH-RH derivative and then concentration of the extract below 25°C;

(15) the process as described in the above (13) or (14), wherein the LH-RH derivative protected with protective group(s) is represented by the formula

5-oxo-Pro-His-Trp-Ser-Tyr-Y-Leu-Arg(X)-Pro-Z

wherein X indicates a protective group, Y indicates a residue selected from DLeu, DAla, DTrp, DSer(tBu), D2Nal and DHis(ImBzl) and Z indicates NH-C₂H₅ or Gly-NH₂, respectively;

(16) purified leuporelin or a salt thereof, wherein the content of total related substances is about 1% or less;

(17) purified leuporelin or a salt thereof, wherein the content of 5-oxo-Pro-D-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NH-CH₂-CH₃ or a salt thereof is about 0.3% or less; and the

like.

Best Mode for Carrying out the Invention

The present invention relates to a process for
5 preparing a LH-RH derivative which comprises subjecting a
solution containing the LH-RH derivative to a step for
treatment with a methacrylic synthetic adsorption resin and
a step for treatment with an aromatic synthetic adsorption
resin, and the like.

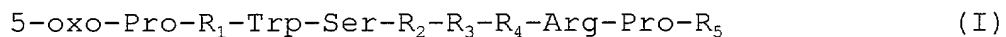
10 Examples of the LH-RH agonist include peptide LH-
RH derivatives possessing LH-RH agonistic activity and
salts thereof and there are, for example, peptide LH-RH
derivatives possessing LH-RH agonistic activity and salts
thereof that are effective against hormone-dependent
15 diseases, particularly sex hormone-dependent cancers (for
example, prostatic cancer, uterine cancer, breast cancer,
pituitary tumor and the like), prostatic hypertrophy,
endometriosis, uterine myoma, precocious puberty,
dysmenorrhea, amenorrhea, premenstrual syndrome and
20 polycystic ovary syndrome, as well as for contraception (or
against infertility if the post-withdrawal rebound effect
is exploited). In addition, they include, for example, LH-
RH derivatives and salts thereof, which are effective
against benign or malignant tumors that are LH-RH-sensitive
25 though being sex hormone-independent, etc.

As for the above-mentioned salt of the LH-RH derivative, a pharmacologically acceptable salt is preferred, and examples of such a salt, in the case where said LH-RH derivative has a basic group such as amino group or the like, include a salt with an inorganic acid (also, designated as an inorganic free acid) (for example, carbonic acid, bicarbonic acid, hydrochloric acid, sulfuric acid, nitric acid, boric acid or the like), a salt with an organic acid (also, designated as an organic free acid) (for example, succinic acid, acetic acid, propionic acid, trifluoroacetic acid or the like) and the like.

Examples of such a salt, in the case where said LH-RH derivative has an acidic group such as carboxyl group or the like, include a salt with an inorganic base (also, designated as an inorganic free base) (for example, an alkali metal such as sodium, potassium, etc., an alkaline earth metal such as calcium, magnesium, etc., or the like), a salt with an organic base (also, designated as an organic free base) (for example, an organic amine such as triethylamine, etc., a basic amino acid such as arginine, etc.) or the like. In addition, said LH-RH derivative may form a metal complex compound (for example, a copper complex, a zinc complex or the like).

As the peptide LH-RH derivative possessing LH-RH agonistic activity, there is, for example, a polypeptide

represented by the formula



wherein R_1 indicates His, Tyr, Trp or $p\text{-NH}_2\text{-Phe}$, R_2 indicates Tyr or Phe and R_3 indicates Gly or a D-amino acid residue that may have a substituent, R_4 indicates Leu, Ile or Nle, R_5 indicates a group represented by the formula Gly-NH- R_6 (wherein R_6 indicates hydrogen atom or an alkyl group that may be substituted) or the formula Gly-NH- R_6 , (wherein R_6 indicates (1) hydrogen atom, (2) an alkyl group that may be substituted with amino group or hydroxyl group) or (3) ureido group ($-\text{NH-CO-NH}_2$), or the like.

In the above-mentioned formula (I), the D-amino acid residue indicated by R_3 is exemplified by an α -D-amino acid having up to 11 carbon atoms (for example, D-Leu, Ile, Nle, Val, Nval, Abu, Phe, Phg, Ser, Thr, Met, Ala or Trp) or the like, each of which may have 1 to 3 adequate substituents (for example, a C_{1-4} alkyl group such as methyl, t-butyl, etc., a C_{1-4} alkoxy group such as t-butoxy, etc., a C_{1-4} alkoxy carbonyl group such as t-butoxycarbonyl, etc., a C_{6-10} aryl group such as 2-naphthyl, etc., an indolyl group or an imidazolyl group, which may be substituted with C_{1-4} alkyl, C_{6-10} aryl or C_{6-10} aryl- C_{1-4} alkyl, respectively, such as indolyl-3-yl, 2-methylindolyl, benzylimidazol-2-yl, etc., or the like). Examples of the substituent of an alkyl group that may be substituted, which is indicated by

R_6 , include hydroxyl or amino. The alkyl group of an alkyl group that may be substituted with amino group or hydroxyl group is exemplified by a C_{1-4} alkyl group and a C_{1-3} alkyl group is especially preferred. Examples of a C_{1-4} alkyl group include methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl or tert-butyl. The number of substituents is, for example, 1 to 3, where 1 to 2 substituents are preferable and one substituent is particularly preferable.

More preferably, examples of the peptide LH-RH derivative possessing LH-RH agonistic activity include a physiologically active peptide represented by the formula (II)

5-oxo-Pro-His-Trp-Ser-Tyr-Y-Leu-Arg-Pro-Z

wherein Y indicates a residue selected from DLeu, DAla, DTrp, DSer(tBu), D2Nal and DHis(ImBzl), and Z indicates $NH-C_2H_5$ or $Gly-NH_2$, respectively, and a salt thereof, etc. Particularly preferable is such a peptide in which Y is Dleu and Z is $NH-C_2H_5$ (namely, the peptide represented by 5-oxo-Pro-His-Trp-Ser-Tyr-DLeu-Leu-Arg-Pro- $NH-C_2H_5$: leuprorelin). As for a salt of the peptide represented by 5-oxo-Pro-His-Trp-Ser-Tyr-DLeu-Leu-Arg-Pro- $NH-C_2H_5$, its acetate (leuprorelin acetate) is particularly preferred among those exemplified as mentioned above.

The abbreviations used herein for denoting amino

acids, peptides, protective groups, etc., in the polypeptides are those according to IUPAC-IUB Commission on Biochemical Nomenclature or conventional codes in this art field, and, also, in the case where an optical isomer may exist with regard to an amino acid, it shall be indicated by the L form, unless otherwise specified.

Examples of the abbreviations are as follows.

	Abu	: Aminobutyric acid
	Aibu	: 2-Aminobutyric acid
10	Ala	: Alanine
	Arg	: Arginine
	Gly	: Glycine
	His	: Histidine
	Ile	: Isoleucine
15	Leu	: Leucine
	Met	: Methionine
	Nle	: Norleucine
	Nval	: Norvaline
	Phe	: Phenylalanine
20	Phg	: Phenylglycine
	Pro	: Proline
	(Pyr)Glu	: Pyroglutamic acid
	Ser	: Serine
	Thr	: Threonine
25	Trp	: Tryptophan

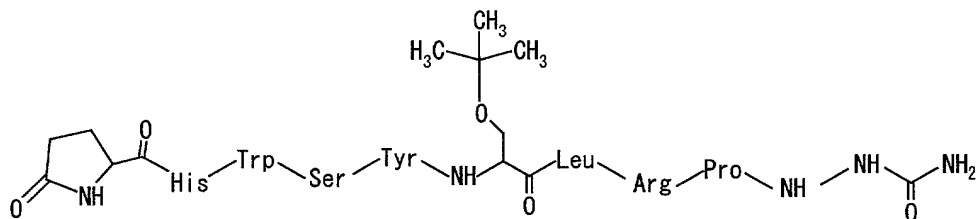
	Tyr	: Tyrosine
	Val	: Valine
	D2Nal	: D-3-(2-Naphthyl)alanine residue
	DSer(tBu)	: O-tert-Butyl-D-serine
5	DHis(ImBzl)	: N ^{im} -Benzyl-D-histidine
	PAM	: Phenylacetamidomethyl
	Boc	: t-Butoxycarbonyl
	Fmoc	: 9-Fluorenylmethyloxycarbonyl
	Cl-Z	: 2-Chlorobenzyloxycarbonyl
10	Br-Z	: 2-Bromobenzyloxycarbonyl
	Bzl	: Benzyl
	Cl ₂ -Bzl	: 2,6-Dichlorobenzyl
	Tos	: p-Toluenesulfonyl
	HONb	: N-Hydroxy-5-norbornene-2,3-dicarboxyimide
15	HOBT	: 1-Hydroxybenzotriazole
	HOObt	: 3-Hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine
	MeBzl	: 4-Methylbenzyl
	Bom	: Benzyloxymethyl
20	Bum	: t-Butoxymethyl
	Trt	: Trityl
	DNP	: Dinitrophenyl
	DCC	: N,N'-Dicyclohexylcarbodiimide

Preferable examples of the peptide LH-RH
 25 derivative possessing LH-RH agonistic activity include, in

addition to the above-mentioned leuprorelin (leuprorelin acetate),

(1) Goserelin

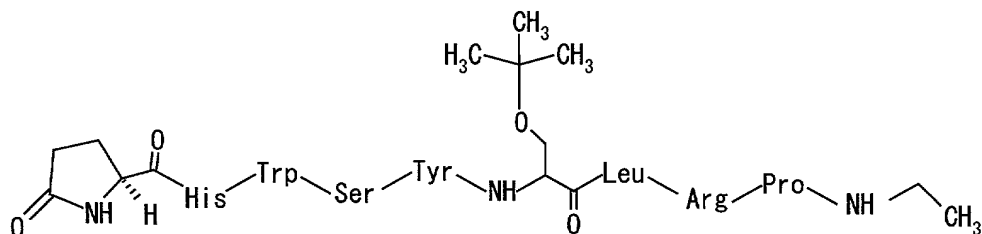
[Chemical formula 1]



(U.S. Patent No. 4,100,274 and JP 52-136172 A),

(2) Buserelin

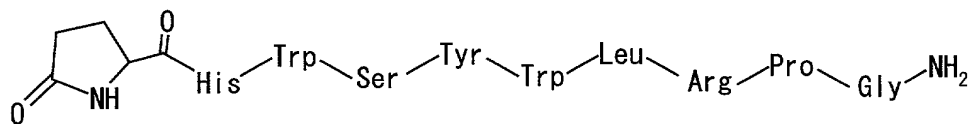
[Chemical formula 2]



(U.S. Patent No. 4,024,248, German Patent No. 2438352 and JP 51-41359 A),

(3) Triptorelin

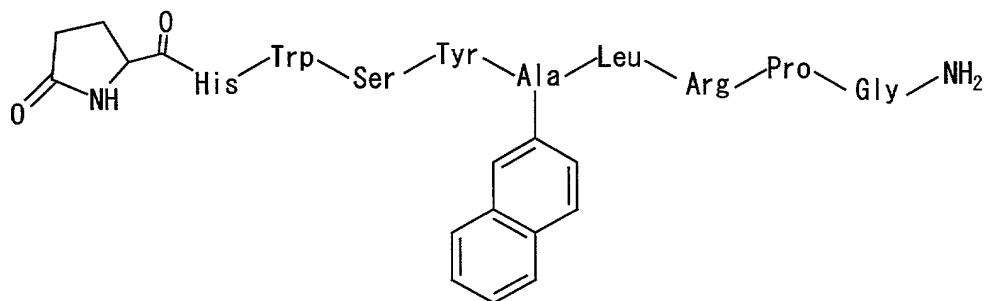
[Chemical formula 3]



(U.S. Patent No. 4,010,125 and JP 52-31073 A),

(4) Nafarelin

[Chemical formula 4]

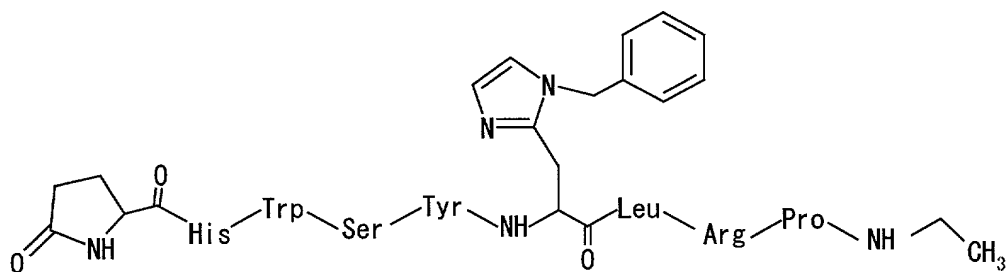


(U.S. Patent No. 4,234,571 , JP 55-164663 A, JP 63-264498 A
and JP 64-25794 A),

5

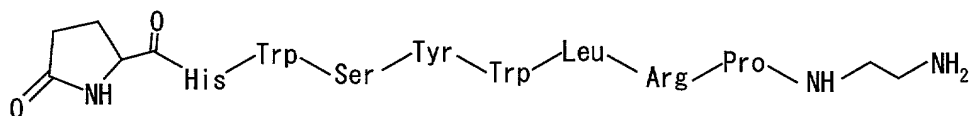
(5) Historelin

[Chemical formula 5]



(6) Deslorelin

[Chemical formula 6]

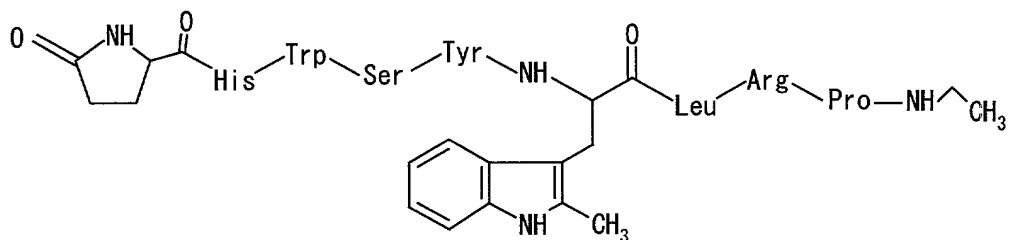


10

(U.S. Patent No. 4,569,967 and U.S. Patent No. 4,218,439),

(7) Meterelin

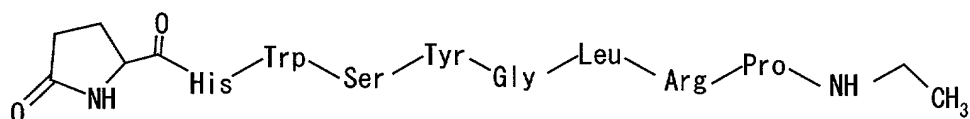
[Chemical formula 7]



(PCT WO 91/18016),

(8) Gonadorelin

5 [Chemical formula 8]



(German Patent No. 2213737) and the like, and salts thereof.

In the above-mentioned [Chemical formula 1] to
[Chemical formula 8], the amino acid corresponding to R₃ in
10 the afore-mentioned formula (I) is in the D-form.

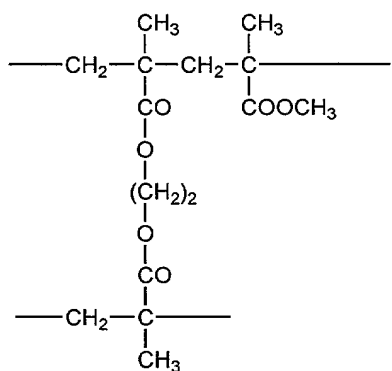
The methacrylic synthetic adsorption resin means
a synthetic adsorption resin of a polymer whose substrate
is a methacrylic acid ester, and racemic isomers, etc. of
the LH-RH derivative can be unexpectedly and effectively
15 removed by subjecting a solution containing the LH-RH
derivative to a step for treatment with said resin
(particularly by the use of an aromatic, synthetic
adsorption resin as described hereinafter in combination)
to prepare (purify) the LH-RH derivative.

20 In addition, racemic isomers, etc. of the LH-RH

derivative can be effectively removed so that a step for treatment with columns in multi stages, which has been heretofore carried out, can be shortened.

Specific examples of the methacrylic synthetic adsorption resin column include HP 2MG (manufactured by Mitsubishi Chemical Corporation), XAD-7 and XAD-8 (manufactured by Organo Company) and the like (preferably, HP 2MG (manufactured by Mitsubishi Chemical Corporation), etc.), but any one may be used as far as it achieves the object to effectively remove racemic isomers, etc. of the LH-RH derivative.

Preferably, the methacrylic synthetic adsorption resin has such particle distribution of the resin that 90% or more of its resin particles have a particle size of 300 μm or more. Also, the methacrylic synthetic adsorption resin having a repeating unit represented by the formula



is preferred.

Particularly, in the case where the above-mentioned leuprorelin (leuprorelin acetate) is prepared

(purified) with the methacrylic synthetic adsorption resin (preferably, HP 2MG (manufactured by Mitsubishi Chemical Corporation), etc.), there can be very effectively removed a racemic isomer at His adjacent to 5-oxo-Pro in leuprorelin (leuprorelin acetate) (hereinafter, abbreviated as D-His² form), a racemic isomer at Trp adjacent to His (hereinafter, abbreviated as D-Trp³ form) and other highly polar related substances.

The above-mentioned "other highly polar related substances" refer to peptide fragments formed by cleavage of the peptide of leuprorelin, reagents used in the reactions and the like, where specific examples include phenol and the like.

The aromatic synthetic adsorption resin (preferably, a styrene-divinylbenzene synthetic adsorption resin) means a synthetic adsorption resin of a porous polymer, which is prepared by copolymerization of styrene and divinylbenzene, where racemic isomers. etc. of the LH-RH derivative can be unexpectedly and effectively removed by subjecting a solution containing the LH-RH derivative to a step for treatment with said resin (particularly by the use of the methacrylic synthetic adsorption resin described above in combination) to prepare (purify) the LH-RH derivative. Specific examples of the aromatic synthetic adsorption resin include HP 20 and HP 21 (manufactured by

Mitsubishi Chemical Corporation), HP 20SS and SP 20SS (manufactured by Mitsubishi Chemical Corporation), XAD-2 and XAD-4 (manufactured by Organo Company) and the like (preferably, HP 20SS (manufactured by Mitsubishi Chemical Corporation) and the like), but any one may be used as far as it achieves the object to effectively remove racemic isomers, etc. of the LH-RH derivative.

Also, it is preferable to use the aromatic synthetic adsorption resin having a particle size of about 60 μm to about 150 μm .

In addition, it is preferable to use the styrene-divinylbenzene synthetic adsorption resin having such particle distribution of the resin that 15% or less of its resin particles have a particle size of 150 μm or more, 70% or more of its resin particles have a particle size of 63 μm or more to 150 μm or less and 20% or less of its resin particles have a particle size of 63 μm or less.

Particularly, in the case where the above-mentioned leuprorelin (leuprorelin acetate) is prepared (purified) by using the aromatic synthetic adsorption resin (preferably, HP 20SS (manufactured by Mitsubishi Chemical Corporation) or the like), there can be very effectively removed D-His² form and L-Leu⁶ form in leuprorelin (leuprorelin acetate) as well as other highly polar, related substances.

The above-mentioned "other highly polar, related substances" has the same meaning as described above.

In order to achieve the above-mentioned object to "effectively remove racemic isomers, etc. of an LH-RH derivative", it is preferable to employ a step for treatment with the methacrylic synthetic adsorption resin and a step for treatment with the aromatic synthetic adsorption resin in combination. In this case, the order of the step for treatment with the methacrylic synthetic adsorption resin and the step for treatment with the aromatic synthetic adsorption resin is not specified particularly in the process for preparing (purifying) the LH-RH derivative, but it is preferable to prepare (purify) the LH-RH derivative by subjecting it to the step for treatment with the methacrylic synthetic adsorption resin and then to the step for treatment with an aromatic, synthetic adsorption resin.

Hereinafter, there is described in detail the process for preparing the LH-RH derivative which comprises subjecting a solution containing the LH-RH derivative to the step for treatment with the methacrylic synthetic adsorption resin and the step for treatment with the aromatic synthetic adsorption resin.

(1) Purification step of LH-RH derivative

[1] The LH-RH derivative before purification

(hereinafter referred to as the crude LH-RH derivative) is dissolved in a buffer solution to prepare the solution containing the LH-RH derivative.

Examples of the buffer solution to be used include, though not being particularly limited as far as it does not inhibit absorption of the LH-RH derivative on the synthetic adsorption resin, water (distilled water or deionized water), an aqueous solution of sodium acetate, an aqueous solution of ammonium acetate, an aqueous solution of sodium phosphate, an aqueous solution of ammonium phosphate, an aqueous solution of ammonium chloride and the like (preferably, an aqueous solution of sodium acetate).

In addition, in order to suppress by-product formation of racemic isomers of the LH-RH derivative, it is preferable to adjust pH of the solution containing the LH-RH derivative at about 4 to about 6, preferably about 4 to about 5, by addition of a pH-adjusting agent (for example, acetic acid, phosphoric acid, hydrochloric acid or the like).

Furthermore, in the case where the LH-RH derivative is the above-mentioned leuprorelin (leuprorelin acetate), it is preferable to control a temperature of the solution containing the LH-RH derivative below 10°C, preferably at about 3°C to about 7°C, in order to prevent by-product formation of a racemic isomer at Trp adjacent to

His in leuprorelin (leuprorelin acetate) (hereinafter, abbreviated as D-Trp³ form) and a racemic isomer at Ser adjacent to Trp in leuprorelin (leuprorelin acetate) (hereinafter, abbreviated as D-Ser⁴ form).

5 In addition, in order to prevent by-product formation of a racemic isomer (specifically, D-Trp³ form in the case of the above-mentioned leuprorelin (leuprorelin acetate)) and highly polar related substances, it is preferable to subject the solution containing the LH-RH derivative promptly after the preparation to the next operation (the step for treatment with the methacrylic synthetic adsorption resin).

10 [2] The solution containing the LH-RH derivative obtained in the above-mentioned item [1] is subjected to the step for treatment with the methacrylic, synthetic adsorption resin.

Specifically, first, the LH-RH derivative is adsorbed on the resin by passing the solution containing the LH-RH derivative obtained in the above [1] through the methacrylic synthetic adsorption resin and then washed with a buffer solution and/or an aqueous solution of an alcohol (for example, an aqueous solution of ethanol, an aqueous solution of methanol, an aqueous solution of n-propanol, an aqueous solution of isopropanol or the like (preferably, an aqueous solution of ethanol or the like)). Examples of the

20

25

buffer solution to be used include water (distilled water or deionized water), an aqueous solution of sodium acetate, an aqueous solution of ammonium acetate, an aqueous solution of sodium phosphate, an aqueous solution of ammonium phosphate, an aqueous solution of ammonium chloride and the like (preferably, an aqueous solution of sodium acetate, an aqueous solution of ammonium acetate or the like). Also, the highly polar related substances (specifically, vide supra) can be effectively removed by washing the resin with an aqueous solution of ethanol. In this case, it is preferable that the concentration of the aqueous solution of ethanol is about 0 to about 20% by volume, preferably about 5 to about 15% by volume.

Next, the LH-RH derivative adsorbed on the resin is eluted with an eluent (for example, an aqueous solution of acetic acid, an aqueous solution of propionic acid, hydrochloric acid, an aqueous solution of phosphoric acid or the like, preferably an aqueous solution of acetic acid or the like). In this case, it is preferable that the concentration of the eluent is about 0.01 M to about 0.50 M, preferably about 0.05 M to about 0.20 M, more preferably about 0.05 M to about 0.10 M. In addition, it is preferable to keep pH of the eluent at neutral or below, preferably about pH 3 to 6. By carrying out the elution under these conditions, it is possible to prevent

effectively by-product formation of racemic isomers (specifically, D-Trp³ form and D-Ser⁴ form in the case of the above-mentioned leuprorelin (leuprorelin acetate)).

Also, it is possible to prevent effectively by-product formation of racemic isomers (specifically, D-Trp³ form and D-Ser⁴ form in the case of the above-mentioned leuprorelin (leuprorelin acetate)) by keeping the operation temperature below 10°C, preferably at about 0°C to about 10°C, more preferably at about 3°C to about 7°C during the procedure of subjecting the solution containing the LH-RH derivative obtained in the above [1] to the step for treatment with the methacrylic, synthetic adsorption resin.

A column packed with the methacrylic, synthetic adsorption resin is employed for the step for treatment with the methacrylic, synthetic adsorption resin.

[3] The solution containing the LH-RH derivative obtained by concentration of the solution containing the LH-RH derivative (the eluate) obtained in the above [2] according to a per se known method is subjected to the step for treatment with the aromatic synthetic adsorption resin.

Specifically, first, the solution containing the LH-RH derivative obtained by concentration of the solution containing the LH-RH derivative (the eluate) obtained in the above [2] according to a per se known method is adsorbed the an aromatic, synthetic adsorption resin and

then washed with a buffer solution. Examples of the buffer solution to be used include, though not being particularly limited as far as it does not inhibit the absorption of the LH-RH derivative on the synthetic adsorption resin, water (distilled water or deionized water), an aqueous solution of sodium acetate, an aqueous solution of ammonium acetate, an aqueous solution of sodium phosphate, an aqueous solution of ammonium phosphate, an aqueous solution of ammonium chloride and the like (preferably, an aqueous solution of sodium acetate or the like). In addition, the washing may be carried out by the use of an aqueous solution of an alcohol such as an aqueous solution of ethanol, an aqueous solution of methanol, an aqueous solution of n-propanol, an aqueous solution of isopropanol or the like (preferably, an aqueous solution of ethanol or the like).

Next, the LH-RH derivative is eluted with an eluent (for example, an aqueous solution of an alcohol such as an aqueous solution of ethanol, an aqueous solution of methanol, an aqueous solution of n-propanol, an aqueous solution of isopropanol or the like (preferably, an aqueous solution of ethanol or the like)). In this case, it is preferable that the concentration of the eluent is about 10% by volume to about 50% by volume, preferably about 15% by volume to about 40% by volume. In addition, it is

preferable to carry out the elution by dividing in several times (preferably, 2 times), and, by carrying out the elution under these preferable conditions, it is possible to remove more effectively racemic isomers (specifically, a racemic isomer at D-Leu adjacent to Trp in leuprorelin (leuprorelin acetate) (hereinafter, abbreviated as L-Leu⁶ form) and the highly polar related substances (specifically, vide supra). In addition, it is preferable that the eluent contains about 0% by volume to about 0.1% by volume of acetic acid, preferably about 0.005% by volume to about 0.01% by volume of acetic acid, and, by carrying out the elution under these preferable conditions, it is possible to prevent effectively by-product formation of racemic isomers (specifically, D-Trp³ form and D-Ser⁴ form in the case of the above-mentioned leuprorelin (leuprorelin acetate)).

Also, in the case where the solution containing the LH-RH derivative obtained by concentration of the solution containing the LH-RH derivative (the eluate) obtained in the above-mentioned item [2] according to a per se known method is subjected to the step for treatment with the aromatic synthetic adsorption resin, the removal of racemic isomers (specifically, a racemic isomer at D-Leu adjacent to Trp in leuprorelin (leuprorelin acetate) (hereinafter, abbreviated as L-Leu⁶ form) and the highly

polar, related substances (specifically, vide supra) becomes more effective by keeping the operation temperature below room temperature, preferably at about 10°C to about 20°C, more preferably at about 13°C to about 17°C.

5 A column packed with the aromatic synthetic adsorption resin is employed for the step for treatment with the aromatic synthetic adsorption resin.

Subsequently, the LH-RH derivative having a very high quality can be obtained in high yield by concentration
10 of the thus-obtained solution containing the LH-RH derivative (the eluate) according to a per se known method.

Although the order of step [1] and step [2] in the purification process of the LH-RH derivative may be reversed, the removal and the by-product formation of
15 racemic isomers (specifically, D-Trp³ form and D-Ser⁴ form in the case of the above-mentioned leuprorelin (leuprorelin acetate)) become more effective by carrying out the step for treatment with the methacrylic synthetic adsorption resin, followed by the step for treatment with the aromatic
20 synthetic adsorption resin.

(2) Treatment step before purification and after deprotection reaction

The process of the present invention relates to that for purifying the LH-RH derivative which comprises
25 subjecting the solution containing the LH-RH derivative to

the step for treatment with the methacrylic, synthetic
adsorption resin and to the step for treatment with the
aromatic synthetic adsorption resin. Also, the present
invention relates to a process for preparing the LH-RH
5 derivative having a higher quality by carrying out a step
for preparing the solution containing the LH-RH derivative,
which is obtained by subjecting the LH-RH derivative
protected with protective group(s) to a deprotection
reaction, prior to the steps for treatment with the
10 synthetic adsorption resins (a step for treatment prior to
the purification) at a low temperature.

Hereinafter, the step for treatment prior to the
purification is described in detail.

[1] Production of crude LH-RH derivative

15 As described hereinabove, the LH-RH derivative
obtained by the process of the present invention is a
peptide, so that a crude LH-RH derivative can be
synthesized according to a per se known method for peptide
synthesis. The method for peptide synthesis may be carried
20 out, for example, either by a solid phase synthesis method
or a liquid phase synthesis method. That is, the objective
peptide can be produced by condensing a partial peptide or
an amino acid, which is capable of constituting the crude
LH-RH derivative, with the residual part and, in the case
25 where the product have a protective group, by eliminating

the protective group. Examples of a known condensation method and the elimination of the protective group include the methods described in the following 1 to 5.

1. M. Bodansky and M. A. Ondetti, Peptide Synthesis, Interscience Publishers, New York (1996)
2. Schroeder and Luebke, The Peptide, Academic Press, New York (1965)
3. Nobuo Izumiya et al. Bases and Experiments for Peptide Synthesis, Maruzen Kabushiki Kaisha (1975)
4. Haruaki Yajima and Syunpei Sakakibara, Lectureships in Biochemistry Experiments 1, Protein Chemistry IV, 205 (1977)
5. Haruaki Yajima, Ed., Continuation of Development of Drugs, Volume 14, Peptide Synthesis, Hirokawa Shoten

For the synthesis of the crude LH-RH derivative, usually, a commercially available resin for a peptide synthesis can be employed. Examples of such a resin can include a chloromethyl resin, a hydroxymethyl resin, benzhydrylamine resin, an aminomethyl resin, a 4-benzyloxybenzyl alcohol resin, a 4-methylbenzhydrylamine resin, a PAM resin, a 4-hydroxymethylmethylphenylacetamidomethyl resin, a polyacrylamide resin, a 4-(2',4'-dimethoxyphenylhydroxymethyl)phenoxy resin, a 4-(2',4'-dimethoxyphenyl-Fmocaminoethyl)phenoxy resin and the like.

By using such a resin, amino acids in which the α -amino group and a functional group at a side chain are appropriately protected, can be condensed on the resin according to the sequence of the objective crude LH-RH derivative by a variety of per se known methods to obtain the LH-RH derivative that is protected with protective groups. After the last reaction, the crude LH-RH derivative peptide is isolated from the resin, and a variety of protective groups are removed at the same time to obtain the objective crude LH-RH derivative.

For condensation of the above-mentioned protected amino acids, a variety of activating reagents for a peptide synthesis can be employed, but carbodiimides are particularly favorable. Examples of carbodiimides to be used include DCC, N,N'-diisopropylcarbodiimide, N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide and the like. In the activation by these reagents, a protected amino acid may be added directly to the resin together with an additive for inhibiting racemization (for example, HOBt, HOObt or HONb), or a protected amino acid may be activated in advance as a symmetric acid anhydride or HOBt ester or HOObt ester and then added to the resin.

A solvent to be used for activation of a protected amino acid and condensation to the resin may be adequately selected from solvents known to be usable in

condensation reactions of peptides. Examples of the solvent to be used include an acid amide such as N,N-dimethylacetamide, N-methylpyrrolidone or the like, a halogenated hydrocarbon such as methylene chloride, chloroform or the like, an alcohol such as trifluoroethanol or the like, a sulfoxide such as dimethyl sulfoxide or the like, pyridine, an ether such as dioxane, tetrahydrofuran or the like, a nitrile such as acetonitrile, propionitrile or the like, an ester such as methyl acetate, ethyl acetate or the like, and an adequate mixture thereof. The reaction temperature is adequately selected from ranges known to be usable in reactions for forming peptide bonds, and usually is adequately selected from a range of about -20°C to about 50°C. The activated amino acid derivative is usually used in a 1.5- to 4-fold excess. In the case where condensation is found to be insufficient as a result of a test using ninhydrin reaction, sufficient condensation can be achieved by repeating the condensation reaction without elimination of protective groups. Even in the case where sufficient condensation cannot be achieved by repeating the reaction, influence on the following reactions can be avoided by acetylation of unreacted amino acids with acetic anhydride or acetylimidazole.

Examples of the protective groups to be used for the amino group in the starting materials include Z, Boc,

t-pentyloxycarbonyl, isobornyloxycarbonyl, 4-methoxybenzyloxycarbonyl, Cl-Z, Br-Z, adamantyloxycarbonyl, trifluoroacetyl, phthaloyl, nitro, formyl, 2-nitrophenylsulfonyl, diphenylphosphinothioyl, Fmoc, p-methoxybenzenesulfonyl and the like.

The carboxyl group may be protected, for example, by an alkyl esterification (for example, the formation of an ester of a straight-chain, branched-chain or cyclic alkyl such as methyl, ethyl, propyl, butyl, t-butyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, 2-adamantyl or the like), an aralkyl esterification (for example, esterification to form the benzyl ester, the 4-nitrobenzyl ester, the 4-methoxybenzyl ester, the 4-chlorobenzyl ester or the benzhydryl ester), phenacyl esterification, the benzyloxycarbonyl hydrazide formation, the t-butoxycarbonyl hydrazide formation, the trityl hydrazide formation or the like.

The hydroxyl group of serine can be protected, for example, by esterification or etherification. Examples of the group suitable for this esterification include a lower (C_{1-6}) alkanoyl group such as acetyl group, etc., an aroyl group such as benzoyl group, etc., a group, which can be derived from carbonic acid, such as benzyloxycarbonyl group, ethoxycarbonyl group, etc., and the like. In addition, examples of a group suitable for the

etherification include benzyl group, tetrahydropyranyl group, t-butyl group and the like.

As for the protective group for phenolic hydroxyl group in tyrosine, for example, Bzl, Cl₂-Bzl, p-nitrobenzyl, Br-Z, t-butyl or the like is used.

As for the protective group for imidazole in histidine, for example, Tos, 4-methoxy-2,3,6-trimethylbenzenesulfonyl, DNP, benzyloxymethyl, Bum, Boc, Trt, Fmoc or the like is used.

Examples of the (crude) LH-RH derivative that is protected with protective group(s) include the derivative in which the α -amino group and a functional group at the side chain of any of the constitutive amino acids in the above-mentioned LH-RH derivative are protected with the above-mentioned protective groups.

Specifically, examples of the LH-RH derivative that is protected with protective groups include a peptide represented by the formula (III)

5-oxo-Pro-His-Trp-Ser-Tyr-Y-Leu-Arg(X)-Pro-Z

wherein X indicates a protective group, Y indicates a residue selected from DLeu, DAla, DTrp, DSer(tBu), D2Nal and DHis(ImBzl) and Z indicates NH-C₂H₅ or Gly-NH₂, respectively, or a salt thereof.

As the protective group represented by X in the formula (III), there can be used Z, Boc, t-

pentyloxycarbonyl, isobornyloxycarbonyl, 4-
 methoxybenzyloxycarbonyl, Cl-Z, Br-Z, adamantyloxycarbonyl,
 trifluoroacetyl, phthaloyl, nitro, formyl, 2-
 nitrophenylsulfonyl, diphenylphosphinothioyl, Fmoc, p-
 5 methoxybenzenesulfonyl and the like, where p-
 methoxybenzenesulfonyl and the like are particularly
 preferable.

Examples of the salt of the peptide represented
 by the formula (III) include salts similar to those
 10 described hereinabove.

In addition, the peptide represented by the
 formula (III) or a salt thereof can be produced by a per se
 known method for peptide synthesis as described hereinabove,
 for example, the peptide can be prepared according to the
 15 method described in WO No. 97/48726 or modification thereof.

As the method for removing the protective groups
 (deprotection reaction), there can be employed, for example,
 hydrogenolysis in a hydrogen stream in the presence of a
 catalyst such as Pd-black, Pd-charcoal or the like; an acid
 20 treatment with anhydrous hydrogen fluoride, methanesulfonic
 acid, trifluoromethanesulfonic acid, trifluoroacetic acid,
 a mixed solution thereof or the like (preferably,
 methanesulfonic acid or the like); a basic treatment with
 diisopropylethylamine, triethylamine, piperidine,
 25 piperazine or the like; reduction with sodium in liquid

ammonia; and the like. The above-mentioned deprotection reaction is carried out in general at a temperature of about 40°C or lower, where, by carrying out the deprotection reaction below about 25°C, preferably at about 5°C to about 15°C, more preferably at about 8°C to about 12°C, the by-product formation of racemic isomers of the LH-RH derivative (specifically, D-His² form in leuprorelin (leuprorelin acetate) in the case of the above-mentioned leuprorelin (leuprorelin acetate)) can be effectively inhibited. In the acid treatment, addition of a cation scavenger such as, for example, anisole, phenol, thioanisole, metacresol, paracresol, dimethyl sulfide, 1,4-butanethiol, 1,2-ethanethiol or the like (preferably, phenol or the like) is effective. Also, 2,4-dinitrophenyl group to be used as the protective group for imidazole in histidine is removed by treatment with thiophenol, and formyl group to be used as the protective group for indole in tryptophan is removed by deprotection by the acid treatment in the presence of the above-mentioned 1,2-ethanethiol, 1,4-butanethiol or the like as well as by an alkaline treatment with a dilute solution of sodium hydroxide, dilute ammonia water or the like.

The reaction time in the above-mentioned deprotection reaction is usually about one hour to about 10 hours, preferably about 2 hours to about 5 hours, more

preferably about 2 hours to about 3 hours.

As for a more specific method for removing the protective groups (deprotection), for example, in the case where the peptide represented by the formula (III) or a salt thereof is subjected to the deprotection reaction, a preferable method is exemplified by an acid treatment with anhydrous hydrogen fluoride, methanesulfonic acid, trifluoromethanesulfonic acid, trifluoroacetic acid, a mixed solution thereof or the like (preferably, methanesulfonic acid or the like). In addition, in this case, it is preferable to add a cation scavenger such as, for example, anisole, phenol, thioanisole, metacresol, paracresol, dimethyl sulfide, 1,4-butanethiol, 1,2-ethanethiol or the like (preferably, phenol or the like). The deprotection reaction is carried out in general at a temperature of about 40°C or less, where, by carrying out the deprotection reaction below about 25°C, preferably at about 5°C to about 15°C, more preferably at about 8°C to about 12°C, the by-product formation of racemic isomers of the LH-RH derivative (specifically, D-His² form in leuprorelin (leuprorelin acetate) in the case of the above-mentioned leuprorelin (leuprorelin acetate)) can be effectively inhibited.

Protection of functional groups which should not participate in the reactions of the starting materials, and

the protective groups as well as the elimination of the protective groups, the activation of the functional groups, which participate in the reactions, and the like may be appropriately selected from known groups or known means.

5 [2] Work-up step after deprotection reaction

First, the solution containing the LH-RH derivative obtained by the deprotection reaction in the above [1] (the reaction solution) is neutralized. The neutralization solution is selected from known solutions, where, for example, in the case where the deprotection reaction is carried out by acid treatment, an aqueous solution of sodium hydroxide, an aqueous solution of potassium hydroxide, an aqueous solution of sodium carbonate, an aqueous solution of sodium hydrogen carbonate, ammonia water, triethylamine or the like (preferably, an aqueous solution of sodium carbonate or the like) is used, and, in the case where the deprotection reaction is carried out by the basic treatment, hydrochloric acid, acetic acid or the like (preferably, hydrochloric acid or the like) is used.

The neutralization reaction is carried out in general at a temperature of about 40°C or less, where, by carrying out the neutralization reaction below about 10°C, preferably below about 5°C, more preferably at about -5°C to about 5°C, the by-product formation of racemic isomers

of the LH-RH derivative (specifically, D-Trp³ form in leuprorelin (leuprorelin acetate) in the case of the above-mentioned leuprorelin (leuprorelin acetate)) can be effectively inhibited.

5 Next, after an organic layer is separated from a neutralized mixture, a buffer solution (for example, an aqueous solution of sodium acetate, an aqueous solution of ammonium acetate, an aqueous solution of ammonium chloride, an aqueous solution of sodium phosphate, an aqueous
10 solution of ammonium phosphate or the like (preferably, an aqueous solution of sodium acetate or the like) is added to transfer the LH-RH derivative into an aqueous layer. In addition, it is preferable to adjust pH at about 3 to about 5, preferably at about 3.9 to about 4.3.

15 The aqueous layer is washed with ethyl acetate, methyl acetate, toluene, methylene chloride, ether or the like (preferably, ethyl acetate or the like) and is concentrated by a per se known method, and pH of the solution is adjusted to about 4 to about 6, preferably to
20 about 4.3 to about 4.7 with a pH-adjusting agent such as acetic acid, hydrochloric acid, phosphoric acid or the like (preferably, acetic acid or the like) to obtain the solution containing the LH-RH derivative.

 A series of steps comprising obtaining the
25 organic layer from the neutralized mixture up to obtaining

the solution containing the LH-RH derivative is carried out in general at a temperature of about 40°C or less, where, by carrying out these steps at about 0°C to about 10°C, preferably at about 3°C to about 7°C, the by-product formation of racemic isomers of the LH-RH derivative (specifically, D-Trp³ form in leuprorelin (leuprorelin acetate) in the case of the above-mentioned leuprorelin (leuprorelin acetate)) can be effectively inhibited.

The LH-RH derivative to be obtained according to the process of the present invention is the LH-RH derivative having an extremely high quality, in which the amount of impurities (racemic isomers of the LH-RH derivative, highly polar related substances and other impurities) are greatly smaller as compared with those in the LH-RH derivative obtained according to the prior art technique. In addition, in the prior art technique, a step for treatment with a synthetic adsorption resin or a step for treatment with an ion-exchange resin has to be repeated several times, whereas, in the present invention, a sufficient purification can be effectively made by carrying out two times of the step for treatment with the synthetic adsorption resin, whereby the LH-RH derivative can be produced in high yields while shortening the operation times. From this point, the process of the present invention is an extremely advantageous process as an

industrial process for preparing the LH-RH derivative, as compared with the prior art technique.

As described hereinabove, the LH-RH derivative having an extremely high quality can be obtained according to the process of the present invention.

"The LH-RH derivative having a high quality" is specifically exemplified by a purified LH-RH derivative (preferably, purified leuprorelin or a salt thereof or the like) and the like, in which the content of total related substances is about 1% or less (preferably, about 0.9% or less, more preferably, about 0.8% or less, further preferably, about 0.7% or less).

Herein, total related substances mean a total of all impurities that are detected by high-performance liquid chromatography and the like, and the impurities are racemic isomers of the LH-RH derivatives, highly polar related substances and other impurities.

More specifically, for example, in the case where the purified LH-RH derivative is purified leuprorelin or a salt thereof, there are exemplified [1] purified leuprorelin or a salt thereof, in which the content of 5-oxo-Pro-D-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NH-CH₂-CH₃ or a salt thereof is about 0.3% or less (preferably, about 0.25% or less, more preferably, about 0.2% or less) and [2] purified leuprorelin or a salt thereof, in which the

content of 5-oxo-Pro-His-Trp-D-Ser-Tyr-D-Leu-Leu-Arg-Pro-NH-CH₂-CH₃ or a salt thereof is about 0.15% or less (preferably, about 0.1% or less, more preferably, about 0%) and the like.

5 The above-mentioned purified LH-RH derivatives are of a low toxicity and can be administered to mammals (for example, human, monkey, dog, rat and mouse) as prophylactics or therapeutics of sex hormone-dependent diseases such as prostatic cancer, benign prostatic
10 hypertrophy, endometriosis, uterine myoma, uterine fibroid tumor, precocious puberty, breast cancer, ovarian cancer, cervical carcinoma and the like or Alzheimer's disease.

Also, any of the above-mentioned purified LH-RH derivatives can be administered orally as a tablet that is
15 coated as needed, a capsule, an elixir, a sustained-release preparation or the like, or can be administered parenterally as an injectable preparation such as a sterile solution in water or another pharmaceutically acceptable solution, or in the preparation form of nasal
20 administration such as a solution, a suspension or the like. The above-mentioned preparation can be manufactured by admixing the above-mentioned purified LH-RH derivative with a physiologically acceptable known carrier, a flavoring agent, an excipient, a vehicle, a preservative, a
25 stabilizer, a binding agent and the like in a unit dosage

form that is required for a generally acceptable pharmaceutical practice.

Examples of additives that can be used for the admixture in a tablet, a capsule and the like include a
5 binding agent such as gelatin, corn starch, tragacanth or gum arabic, an excipient such as crystalline cellulose, a swelling agent such as corn starch, gelatin or alginic acid, a lubricant such as magnesium stearate, a sweetener such as sucrose, lactose or saccharin, a flavoring agent such as
10 peppermint, Akamono oil or cherry, and the like. In the case where the dosage unit form is a capsule, a liquid carrier such as an oil and fat can be further contained in addition to the above-mentioned type of materials. A sterile composition for injection can be formulated
15 according to a conventional pharmaceutical practice that is carried out by dissolving or suspending an active substance, a naturally occurring vegetable oil such as sesame oil, coconut oil or the like, and the like in a vehicle such as water for injection. An aqueous solution for injection to
20 be used is exemplified by physiological saline, an isotonic solution containing glucose and other auxiliary agents (for example, D-sorbitol, D-mannitol, sodium chloride and the like), where an adequate solubilizing agent such as an alcohol (for example, ethanol), a polyalcohol (for example, propylene glycol and polyethylene glycol), a non-ionic,
25

surface active agent (for example, polysorbate 80 (TM) and HCO-50) and the like can be used in combination. An oily liquid to be used is exemplified by sesame oil, soybean oil or the like, where a solubilizing agent such as benzyl benzoate, benzyl alcohol or the like can be used in combination.

Also, the above-mentioned preparations may be compounded with a buffering agent (for example, a phosphate buffer solution or a sodium acetate buffer solution) an analgesic (for example, benzalkonium chloride, procaine hydrochloride or the like), a stabilizer (for example, human serum albumin, polyethylene glycol or the like), a preservative (for example, benzyl alcohol, phenol or the like), an antioxidant and the like. The injectable solution thus prepared usually is filled in adequate ampoules.

A sustained-release preparation comprising the above-mentioned purified LH-RH derivative, can be manufactured according to a per se known method such as, for example, the method described in JP 60-100516 A, JP 62-201816 A, JP 4-321622 A, JP 6-192068 A, JP 9-132524 A, JP 9-221417 A, JP 11-279054 A, WO 99/360099 or the like.

The dosage of the above-mentioned purified LH-RH derivative varies widely depending on subject disease, subject animal and the like, but the dosage per once can be

appropriately selected, for example, preferably from a range of about 0.01 mg to 100 mg/kg body weight for an adult patient of prostatic cancer. More preferably, the dosage can be appropriately selected from a range of about 0.05 mg to 50 mg/kg body weight.

The dosage of the sustained-release preparation containing said above-mentioned purified LH-RH derivative, for example, in the case of a sustained-release preparation having drug efficacy for one month, the dosage of the sustained-release preparation per once can be appropriately selected preferably from a range of about 0.1 mg to 500 mg/kg weight for an adult patient of prostatic cancer. More preferably, the dosage can be appropriately selected from a range of about 0.2 mg to 300 mg/kg body weight.

Examples

The following examples illustrate the present invention in more detail, but they are not intended to restrict the scope of the present invention.

Example 1 Preparation of 5-oxo-L-prolyl-L-histidyl-L-tryptophyl-L-seryl-L-tyrosyl-D-leucyl-L-leucyl-L-arginyl-N-ethyl-L-prolinamide (leuprorelin, leuprolide) (1)

To a mixed solution of 748 g of methanesulfonic acid and 56 g of phenol was added 68.35 g (52.92 g as pure

leuprolide) of 5-oxo-L-prolyl-L-histidyl-L-tryptophyl-L-seryl-L-tyrosyl-D-leucyl-L-leucyl-N^m-p-methoxybenzenesulfonyl-L-arginyl-N-ethyl-L-prolinamide, and the resulting solution was reacted at 8 to 12°C for about 3 hours. The reaction solution was added at -5 to 5°C into a mixed solution of a solution of potassium carbonate (646 g) in water (1896 mL) and 396 mL of ethyl acetate, and the resulting mixture was then stirred at 3 to 7°C for 30 minutes and neutralized. The organic layer was separated, mixed with 2316 mL of a 0.11 M sodium acetate buffer solution (pH: 3.9 to 4.3) and stirred at 3 to 7°C to carry out the extraction. The aqueous layer was adjusted to pH 4.3 to 4.7 with acetic acid and mixed with 372 mL of ethyl acetate, and the resulting layers were then separated. The aqueous layer was washed at 3 to 7°C with 372 mL of ethyl acetate. The aqueous layer was concentrated under reduced pressure below 25°C and then adjusted to pH 4.3 to 4.7 with acetic acid to obtain an aqueous solution (1) of leuprolide.

Yield of leuprolide acetate: 44.26 g (90.9%)

Quality by high-performance liquid chromatography (peak area percentage for leuprolide): D-Trp³ form, not detected; D-Ser⁴ form, not detected; D-His² form, 0.27%; L-Leu⁶ form, 1.14%

Example 2 Preparation of 5-oxo-L-prolyl-L-

histidyl-L-tryptophyl-L-seryl-L-tyrosyl-D-leucyl-L-leucyl-L-arginyl-N-ethyl-L-prolinamide (leuprolide) (2)

Through a column packed with 5500 mL of a methacrylic, synthetic adsorption resin (HP 2MG: Mitsubishi Chemical Corporation) was passed at 3 to 7°C 5600 g of the aqueous solution (1) of leuprolide (leuprolide acetate: 85.46 g). The resulting column was washed by passing successively 11000 mL of a 0.3 M aqueous solution of sodium acetate (pH: 6.3), 13750 mL of a 0.025 M aqueous solution of ammonium acetate and 19250 mL of a 10% by volume, aqueous solution of ethanol at 3 to 7°C. The elution was carried out by passing through 41250 mL of a 0.05 M aqueous solution of acetic acid at 3 to 7°C. Fractions containing leuprolide were collected and concentrated under reduced pressure below 35°C to obtain an aqueous solution (2) of leuprolide.

Yield of leuprolide acetate: 73.77 g (86.3%)

Quality by high-performance liquid chromatography (peak area percentage for leuprolide): D-Trp³ form, 0.11%; D-Ser⁴ form, 0.03%; D-His² form, 0.24%; L-Leu⁶ form, 0.42%.

Example 3 Preparation of 5-oxo-L-prolyl-L-histidyl-L-tryptophyl-L-seryl-L-tyrosyl-D-leucyl-L-leucyl-L-arginyl-N-ethyl-L-prolinamide (leuprolide) (3)

Through a column packed with 2200 mL of small

particles of an aromatic, synthetic adsorption resin (HP 20SS: Mitsubishi Chemical Corporation) was passed at 13 to 17°C 3731 g of the aqueous solution (2) of leuprolide (leuprolide acetate: 73.54 g). The resulting column was washed by passing successively 6600 mL of a 0.1 M aqueous solution of sodium acetate (pH: 6.3), 9900 mL of a 0.01 M aqueous solution of ammonium acetate and 2200 mL of water at 13 to 17°C. The elution was carried out by passing through successively 8800 mL of a 20% by volume, aqueous solution of ethanol and 8800 mL of a 35% by volume, aqueous solution of ethanol at 13 to 17°C. Fractions containing leuprolide were collected and concentrated under reduced pressure below 35°C to obtain an aqueous solution (3) of leuprolide.

Yield of leuprolide acetate: 64.55 g (87.8%)

Quality by high-performance liquid chromatography (peak area percentage for leuprolide): D-Trp³ form, 0.12%; D-Ser⁴ form, 0.04%; D-His² form, 0.18%; L-Leu⁶ form, not detected.

Example 4 Preparation of 5-oxo-L-prolyl-L-histidyl-L-tryptophyl-L-seryl-L-tyrosyl-D-leucyl-L-leucyl-L-arginyl-N-ethyl-L-prolinamide monoacetate (leuprolide acetate) (4)

Column chromatography of 436.7 g of the aqueous

solution (3) of leuprolide (leuprolide acetate: 63.90 g) was carried out by using 2350 mL of cross-linked dextran gel (Sephadex LH-20: Pharmacia) with elution with a 0.005 M aqueous solution of acetic acid. Fractions containing leuprolide were collected and subjected to decolorization with 1.16 g of activated carbon. The activated carbon was filtered, the filtrate was concentrated under reduced pressure below 35°C and the concentrate was subjected to ultrafiltration. The filtrate was lyophilized to obtain 60.38 g of freeze-dried leuprolide acetate.

Yield of leuprolide acetate: 60.02 g (98.9%)

Quality by high-performance liquid chromatography (peak area percentage for leuprolide): D-Trp³ form, 0.11%; D-Ser⁴ form, 0.03%; D-His² form, 0.18%; L-Leu⁶ form, not detected.

Example 5 Preparation of 5-oxo-L-prolyl-L-histidyl-L-tryptophyl-L-seryl-L-tyrosyl-D-leucyl-L-leucyl-L-arginyl-N-ethyl-L-prolinamide (leuprolide) (3')

Through a column packed with 120 L of small particles of an aromatic, synthetic adsorption resin (HP 20SS: Mitsubishi Chemical Corporation) was passed at 13 to 17°C 200 L of the aqueous solution (2) of leuprolide (leuprolide acetate: 4080 g). The resulting column was washed by passing successively 360 L of a 0.1 mol/L aqueous

solution of sodium acetate (pH: 6.3), 540 L of a 0.01 mol/L aqueous solution of ammonium acetate and 120 L of water at 13 to 17°C. The elution was carried out by passing through successively 960 L of a 15% by volume, aqueous solution of ethanol (containing 0.01 W/V% of acetic acid), 480 L of a 20% by volume, aqueous solution of ethanol (containing 0.01 W/V% of acetic acid) and 360 L of a 30% by volume, aqueous solution of ethanol (containing 0.01 W/V% of acetic acid) at 13 to 17°C. Fractions containing leuprolide were collected and concentrated under reduced pressure below 35°C to obtain an aqueous solution of leuprolide.

Yield of leuprolide acetate: 3527 g (86.4%)

Quality by high-performance liquid chromatography (peak area percentage for leuprolide): D-Trp³ form, 0.13%; D-Ser⁴ form, 0.04%; D-His² form, 0.25%; L-Leu⁶ form, not detected.

Industrial Applicability

According to the process of the present invention, it becomes possible to suppress the by-product formation of impurities such as racemic isomers of LH-RH derivatives and to effectively remove the impurities such as the racemic isomers, so that it becomes possible to produce the LH-RH derivatives having an extremely high quality. Also, according to the process of the present invention, a

sufficient purification can be made effectively by carrying out two times of a step for treatment with a synthetic, adsorption resin, whereby the LH-RH derivatives can be produced in high yields effectively by easy operations and without involving any complicated procedure for solid-liquid separation.

100-200-300-400-500-600-700-800-900-1000

What is claimed is:

1. A process for preparing a LH-RH derivative which comprises subjecting a solution containing the LH-RH derivative to a step for treatment with a methacrylic synthetic adsorption resin and a step for treatment with an aromatic synthetic adsorption resin.

2. The process according to claim 1, wherein the LH-RH derivative is a peptide represented by the formula

5-oxo-Pro-His-Trp-Ser-Tyr-Y-Leu-Arg-Pro-Z

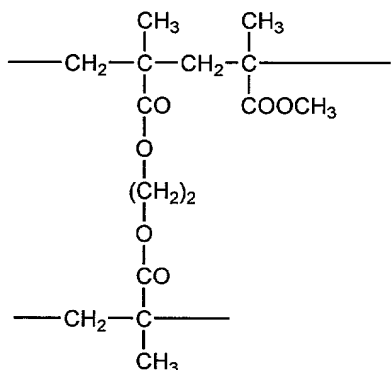
wherein Y indicates a residue selected from DLeu, DAla, DTrp, DSer(tBu), D2Nal and DHis(ImBzl), and Z indicates NH-C₂H₅ or Gly-NH₂, respectively, or a salt thereof.

3. The process according to claim 1, wherein the LH-RH derivative is a peptide represented by the formula

5-oxo-Pro-His-Trp-Ser-Tyr-DLeu-Leu-Arg-Pro-NH-C₂H₅

or its acetate.

4. The process according to claim 1, wherein said process comprises using a methacrylic synthetic adsorption resin having a repeating unit represented by the formula



5. The process according to claim 1, wherein the aromatic synthetic adsorption resin is a styrene-divinylbenzene synthetic adsorption resin.

6. The process according to claim 5, wherein an average particle size of the styrene-divinylbenzene, synthetic adsorption resin is about 60 μm to about 150 μm .

7. The process according to claim 1, wherein said process comprises subjecting a solution containing the LH-RH derivative to the step for treatment with a methacrylic synthetic adsorption resin below about 10°C.

8. The process according to claim 1, wherein said process comprises subjecting a solution containing the LH-RH derivative to the step for treatment with an aromatic synthetic adsorption resin at about 10°C to about 20°C.

9. The process according to claim 1, wherein said process comprises subjecting a solution containing the LH-RH derivative to the step for treatment with a methacrylic, synthetic adsorption resin, followed by

subjecting to the step for treatment with an aromatic, synthetic adsorption resin.

10. The process according to claim 1, said process comprises passing a solution containing the LH-RH
5 derivative through a resin in the step for treatment with a methacrylic synthetic adsorption resin and then eluting the LH-RH derivative, which is adsorbed on the resin, with an aqueous solution of acetic acid.

11. The process according to claim 10, wherein
10 the concentration of an aqueous solution of acetic acid is about 0.01 M to about 0.50 M.

12. The process according to claim 1, wherein said process comprises passing a solution containing the LH-RH derivative through a resin in the step for treatment
15 with a methacrylic, synthetic adsorption resin, followed by washing with an aqueous solution of ethanol, and then by eluting the LH-RH derivative that is adsorbed on the resin.

13. The process according to claim 1, wherein a solution containing the LH-RH derivative is that obtained
20 by subjecting the LH-RH derivative protected with protective group(s) to a deprotection reaction followed by a neutralization reaction below about 10°C.

14. The process according to claim 1, wherein a solution containing the LH-RH derivative is that obtained
25 by subjecting the LH-RH derivative protected with

protective group(s) to a deprotection reaction and then a neutralization reaction below about 10°C, followed by subjecting the resulting mixture to extraction of the LH-RH derivative and then concentration of the extract below 25°C.

5 15. The process according to claim 13 or 14, wherein the LH-RH derivative protected with protective group(s) is represented by the formula

5-oxo-Pro-His-Trp-Ser-Tyr-Y-Leu-Arg(X)-Pro-Z

10 wherein X indicates a protective group, Y indicates a residue selected from DLeu, DAla, DTrp, DSer(tBu), D2Nal and DHis(ImBzl) and Z indicates NH-C₂H₅ or Gly-NH₂, respectively.

15 16. Purified leuprorelin or a salt thereof, wherein the content of total related substances is about 1% or less.

 17. Purified leuprorelin or a salt thereof, wherein the content of 5-oxo-Pro-D-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NH-CH₂-CH₃ or a salt thereof is about 0.3% or less.

Attorney's Docket No. _____

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that: My residence, post office address and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (if only one name is listed at 201) below or an original, first and joint inventor (if plural names are listed at 201-208 below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PROCESS FOR PREPARING LH-RH DERIVATIVES

which is described and claimed in:

- ☐ the specification attached hereto.
- ☐ the specification in U.S. Application Serial Number _____, filed on _____.
- ☒ the specification in PCT international application Number PCT/JP00/04277,
filed on June 29, 2000; and was amended on _____.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a). I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

[illegible]

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below, and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose material information as defined in 37 CFR §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

Prior U.S. Applications or PCT International Applications Designating the U.S-Benefit Under 35 U.S.C. §120					
U.S. Applications			Status (Check One)		
Application Serial No.	U.S. Filing Date	Patented	Pending	Abandoned	
PCT Applications Designating the U.S.					
Application No.	Filing Date	U.S. Serial No. Assigned			

CLAIM FOR BENEFIT OF PRIOR U.S. PROVISIONAL APPLICATION(S)
(35 U.S.C. § 119(e))

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below:

Applicant	Provisional Application Number	Filing Date

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) with full powers of association, substitution and revocation to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Sewall P. Bronstein (Reg. No. 16,919)
David G. Conlin (Reg. No. 27,026)
George W. Neuner (Reg. No. 26,964)
Ernest V. Linek (Reg. No. 29,822)

Linda M. Buckley (Reg. No. 31,003)
Ronald I. Eisenstein (Reg. No. 30,628)
Henry D. Pahl, Jr. (Reg. No. 20,438)
Peter J. Manus (Reg. No. 26,766)

David S. Resnick (Reg. No. 34,235)
Peter F. Corless (Reg. No. 33,860)

SEND CORRESPONDENCE TO: <u>Dike, Bronstein, Roberts & Cushman, LLP</u> <u>130 Water Street</u> <u>Boston, Massachusetts 02109</u>	DIRECT TELEPHONE CALLS TO: <u>(617) 523-3400</u>
---	--

2 0 0	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE OR COUNTRY AND ZIP CODE
		<u>SASAKI</u>	<u>Yasuhiro</u>	
		<u>Hikari-shi</u> <u>SPX</u>	<u>Yamaguchi</u>	<u>Japan</u> ✓
		<u>9-8, Senboudai 3-chome</u>	<u>Hikari-shi, Yamaguchi, Japan</u>	

2 0 0	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE OR COUNTRY AND ZIP CODE
		<u>SHIMIZU</u>	<u>Katsuji</u>	
		<u>Kumage-gun</u> <u>SPX</u>	<u>Yamaguchi</u>	<u>Japan</u> ✓
		<u>821-3, Yashiro, Kumage-cho</u>	<u>Kumage-gun, Yamaguchi, Japan</u>	

	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE OR COUNTRY AND ZIP CODE

2 0 4	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE OR COUNTRY AND ZIP CODE

2 0 5	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE OR COUNTRY AND ZIP CODE

2 0 6	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE OR COUNTRY AND ZIP CODE

2 0 7	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE OR COUNTRY AND ZIP CODE

2 0 8	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE OR COUNTRY AND ZIP CODE

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signature of Inventor 201 <i>Y. Sasake</i>	Signature of Inventor 202 <i>H. Shimizu</i>
Date: November 20, 2001	Date: November 20, 2001